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A RhoGEF and Rho Family GTPase-Activating Protein Complex Links the Contractile Ring to Cortical Microtubules at the Onset of Cytokinesis

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Summary

The mechanism that positions the cytokinetic contractile ring is unknown, but derives from the spindle midzone. We show that an interaction between the Rho GTP exchange factor, Pebble, and the Rho family GTPase-activating protein, RacGAP50C, connects the contractile ring to cortical microtubules at the site of furrowing in *D. melanogaster* cells. Pebble regulates actomyosin organization, while RacGAP50C and its binding partner, the Pavarotti kinesin-like protein, regulate microtubule bundling. All three factors are required for cytokinesis. As furrowing begins, these proteins colocalize to a cortical equatorial ring. We propose that RacGAP50C-Pavarotti complexes travel on cortical microtubules to the cell equator, where they associate with the Pebble RhoGEF to position contractile ring formation and coordinate F-actin and microtubule remodeling during cytokinesis.

Introduction

Cytokinesis, the final step in cell division, involves the formation and constriction of an actomyosin-based contractile ring. In *Drosophila melanogaster* embryonic epithelial cells, constriction occurs during anaphase B and telophase to generate two daughter cells, each containing one set of the recently separated sister chromatids. Constriction of the cleavage furrow proceeds through the activity of the myosin II motor protein acting on an F-actin network (reviewed by Glotzer, 2001). Members of the Rho subfamily of small G proteins are potent regulators of the actin cytoskeleton in a variety of contexts (reviewed by Hall, 1998). Formation of the actomyosin network during cytokinesis requires Rho1 (Prokopenko et al., 1999; O'Connell et al., 1999; Jantsch-Plunger et al., 2000). Like all small G proteins, Rho1 is active when GTP is bound and inactive when GDP is bound. Activation is mediated by guanine nucleotide exchange factors (GEFs) that catalyze the displacement of GDP and the uptake of GTP, whereas inactivation is regulated by GTPase-activating proteins (GAPs) that stimulate the intrinsic GTPase activity of the G protein.

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Molecular and genetic studies have shown that the *D. melanogaster* RhoGEF, Pebble (PBL), and its mammalian ortholog, the protooncogene ECT2, are required for cytokinesis (Prokopenko et al., 1999; Tatsumoto et al., 1999). *pbl* mutant embryos proceed normally through the first 13 syncytial mitotic cycles following fertilization and cellularize normally during G2 phase of cycle 14, but they fail to undergo cytokinesis during the fourteenth and subsequent division cycles (Hime and Saint, 1992; Lehner, 1992). PBL binds to Rho1, but not Rac1 or Cdc42, and sensitized *pbl* mutant alleles show strong genetic interactions with *Rho1* but not *Rac1* or *Cdc42* alleles (Prokopenko et al., 1999). During cytokinesis in epithelial cells of the embryo, PBL accumulates in the contractile ring during furrowing, where it appears to stimulate Rho1-mediated organization and activity of the actomyosin contractile ring (Prokopenko et al., 1999).

Reorganization of the actomyosin contractile apparatus occurs coincident with reorganization of the microtubule network. During anaphase, the mitotic spindle is remodeled to form a midzone bundled microtubule structure referred to as the central spindle, which is further compacted into a late cytokinetic structure termed the midbody (reviewed by Glotzer, 2001). Curiously, another regulator of Rho family G protein activity, the *Caenorhabditis elegans* CYK-4 GAP, is required for microtubule bundling, as microtubule reorganization fails in *cyk-4* mutant embryos. It also fails in embryos mutant for the *zen-4/CeMKLP1* gene, which encodes a kinesin-like protein that forms a complex with CYK-4. This complex has been shown to bundle microtubules in vitro (Mishima et al., 2002). The CYK-4 and ZEN-4 proteins and their respective mammalian orthologs localize to the central spindle and are all essential for cytokinesis (Jantsch-Plunger et al., 2000; Hirose et al., 2001; Kuriyama et al., 2002; Raich et al., 1998), as is Pavarotti (PAV), the *D. melanogaster* ortholog of ZEN-4 (Adams et al., 1998).

It is not known how remodeling of the microtubule and F-actin networks is coordinated during cytokinesis. Although initial studies focused on their role in F-actin remodeling, recent studies have now linked Rho family members to microtubule organization. For example, depolymerization of microtubules results in an increase in the amount of active RhoA and the formation of contractile actin bundles, while microtubule polymerization results in an increase in the amount of active Rac1 and the formation of lamellipodia (Ren et al., 1999; Waterman-Storer et al., 2000). RhoA can also mediate selective microtubule stabilization (Cook et al., 1998), as can the RhoA effector mDia (Ishizaki et al., 2001; Palazzo et al., 2001), while the Rac1/Cdc42 effector PAK is capable of activating the microtubule destabilizer Stathmin (Daub et al., 2001). It is possible, therefore, that Rho family members play roles in both F-actin and microtubule organization during dynamic processes such as cytokinesis. An important but poorly understood aspect of the relationship between the microtubule and F-actin networks is the nature of the signal that positions the

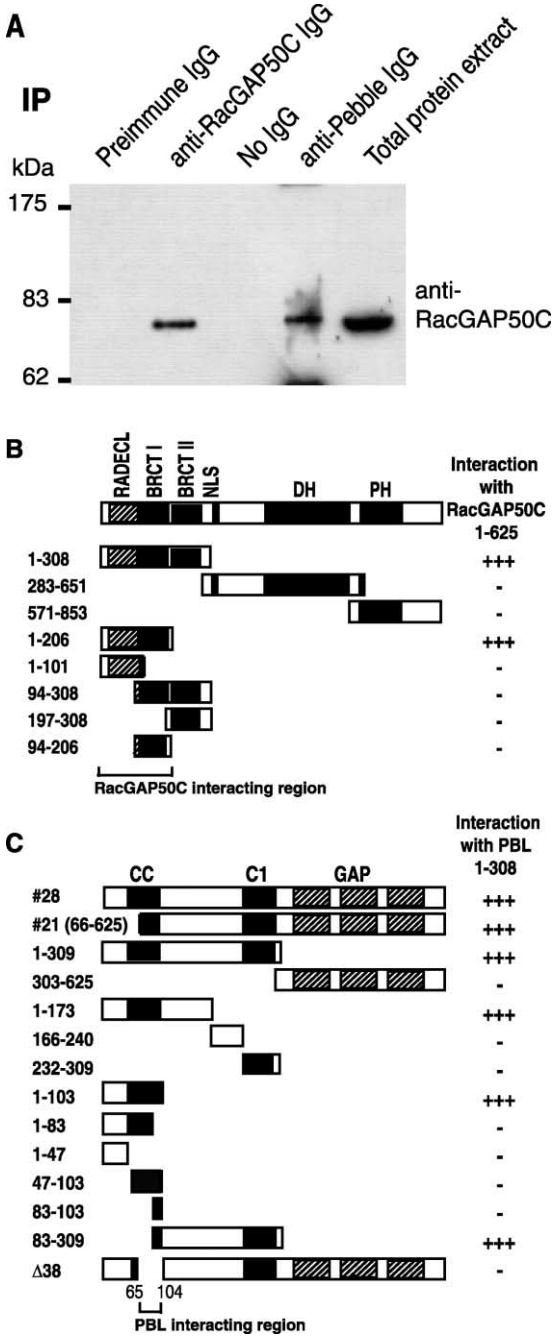


Figure 1. Identification and Characterization of a Pebble-RacGAP50C Interaction

(A) RacGAP50C coimmunoprecipitates with PBL from a 0–5 hr embryonic extract. Samples were immunoprecipitated with the antibodies indicated above each well, or with no antibodies (no IgG), before being electrophoresed. The right-hand lane was loaded with total embryonic protein extract. Following Western blotting, RacGAP50C was detected using anti-RacGAP50C antibodies.

(B and C) Schematic representation of yeast two-hybrid assay results with PBL and RacGAP50C. Domains are labeled as follows: RADECL: Rad4/Ect2-like domain; BRCT: BRCA1 C-terminal domain; NLS: nuclear localization sequence; DH: Dbl homology domain; PH: pleckstrin homology domain; GAP: GTPase-activating domain; C1: phorbol ester/diacylglycerol binding domain; CC: coiled-coil domain. The results of the interactions are shown on the right, with +++ representing a strong interaction and − indicating no interaction.

contractile ring and initiates furrowing. It is now generally accepted that the signal originates from the midzone of the anaphase microtubule network (Cao and Wang, 1996; Wheatley and Wang, 1996), although the nature of the stimulus is unknown.

We report here the identification of a complex between two Rho family regulators, the RhoGEF PBL and RacGAP50C, the *D. melanogaster* ortholog of the CYK-4 Rho family GAP. We show that a ring of RacGAP50C and PAV associated with cortical microtubules colocalizes with PBL in dividing embryonic epithelial cells, forming a link between the actomyosin and microtubule networks. These observations suggest a molecular model for contractile ring positioning and function whereby RacGAP50C-PAV complexes, positioned at the equatorial cortex of the cell by their association with microtubules, interact with cortical PBL to activate Rho1, initiate formation of the contractile ring, and coordinate F-actin and microtubule dynamics during furrowing.

Results

The Pebble RhoGEF and RacGAP50C Rho Family GAP Interact

An N-terminal region of PBL (Figure 1A, PBL_{1-308}), which contains several conserved domains, was used as a bait in a yeast two-hybrid screen of 1.5×10^6 cDNA clones from a 0–4 hr *D. melanogaster* embryonic library. The two clones that showed the strongest interaction encoded the *D. melanogaster* RacGAP50C (data not shown), a Rho family GAP (Sotillos and Campuzano, 2000). To test whether this interaction occurs in vivo, we generated polyclonal anti-RacGAP50C antibodies and used them in coimmunoprecipitation experiments. The anti-RacGAP50C was found to precipitate RacGAP50C (Figure 1A, lane 2) and to detect RacGAP50C in early embryonic extracts (Figure 1A, lane 5). This band was not detected when the preimmune IgG was used (Figure 1A, lane 1) nor when protein extracted from RacGAP50C RNAi-treated Schneider line 2 cells was used (data not shown). Immunoprecipitation with anti-PBL IgG (Prokopenko et al., 1999) also precipitated the ~70 kDa RacGAP50C protein (Figure 1A, lane 4), indicating the presence of a PBL-RacGAP50C complex in embryonic tissues. We also observed a strong interaction between the human PBL and RacGAP50C orthologs, ECT2 and MgcRacGAP, in a yeast two-hybrid assay (data not shown), suggesting that the interaction has been conserved during animal evolution.

A Conserved N-Terminal BRCT Domain-Containing Region Is Required for PBL-RacGAP50C Interaction

Using the yeast two-hybrid assay, we dissected the regions of PBL and RacGAP50C that are necessary for the interaction. PBL constructs fused to the LexA DNA

(B) The first BRCT domain and the extended N-terminal region of homology, termed the RADECL domain, are necessary for an interaction with full-length RacGAP50C.

(C) The coiled-coil domain of RacGAP50C is necessary for the interaction with PBL.

binding domain were tested for an interaction with full-length RacGAP50C fused to the VP16 activation domain (Figure 1B). The region of PBL used in the original screen contains two consensus BRCT (BRCA1 C-terminal) domains and a conserved sequence, here termed the RADECL (RAD4/ECT2-like) domain, immediately N-terminal to the first BRCT domain. The RADECL domain is found in PBL and its orthologs and in the BRCT domain-containing proteins Mus101 and Topoisomerase II binding protein/RAD4-like. It is invariably N-terminal to and contiguous with a BRCT domain, with which it appears to have coevolved (A. Harley and R.S., unpublished data). A construct containing the RADECL and first BRCT domains ($PBL_{1–206}$) interacted with full-length RacGAP50C as strongly as the original construct. A variety of other domains and subdomains (summarized in Figure 1A) failed to interact, showing that the first BRCT domain of PBL and its associated conserved N-terminal extension, the RADECL domain, are necessary for the interaction with RacGAP50C.

Sequential rounds of interaction assays using the original PBL bait, $PBL_{1–308}$, and various RacGAP50C constructs refined the interaction domain to the coiled-coil region (Figure 1C, CC) of RacGAP50C ($RacGAP50C_{1–103}$). The Δ38 RacGAP50C construct, deleted for amino acids 66–103, abolished all interactive activity with PBL, showing that residues required for the interaction with PBL lie within this region.

Drosophila RacGAP50C Is Essential for Cytokinesis

To examine the effect of removing RacGAP50C, we used RNA interference (RNAi) to reduce the level of endogenous protein in *D. melanogaster* Schneider line 2 (S2) cells (Clemens et al., 2000). Treatment with dsRNA corresponding to the GAP domain-encoding region of *RacGAP50C* resulted in the majority (75%–80%) of S2 cells becoming multinucleate within 2 days (Figure 2A). This strong multinucleate phenotype was also seen with dsRNA directed toward *pbl* and *Rho1*, but not toward *Rac1*, *Rac2*, *Cdc42*, *mtl*, or *RhoL* (data not shown).

We also generated an inducible *RacGAP50C* RNAi construct under the control of the *Gal4* upstream activation sequence (UAS; Brand and Perrimon, 1993). *engrailed::GAL4* (*en::GAL4*)-induced expression of $RacGAP50C^{RNAi}$ resulted in abnormally large posterior pupal wing cells (Figure 2E compared with Figure 2D) that were typically binucleate (Figures 2C and 2E compared with Figures 2B and 2D). Expression of the RNAi construct had other consequences, for example on the number of trichome hairs per cell (Figure 2E compared with Figure 2D). However, planar polarity appeared to be only mildly disrupted. These aspects of the *RacGAP50C* phenotype are not discussed further here.

In order to further characterize the nature of the cytokinetic defect, we observed live *Drosophila* S2 cells that had been treated with RacGAP50C RNAi and maintained for 24 hr (Figures 2F and 2G). The vital dyes, Hoechst 33258 and SynaptoRed, were used to stain DNA and membranes, respectively. Untreated S2 cells (Figure 2F), or S2 cells treated with a control RNAi directed against GFP (data not shown), were round during metaphase. As anaphase proceeded, the cells elongated and then exhibited considerable blebbing immediately preceding and during cytokinesis. Mitotic RacGAP50C RNAi-treated cells were also round until anaphase, when they elongated. Although cells exhibited apparently random dynamic shape changes at this time, there was considerably less blebbing, and there was no evidence of stable furrow formation during successful mitoses (Figure 2G).

RacGAP50C and *pbl* Interact Genetically

We used the RNAi-induced phenotype to test for *pbl-RacGAP50C* genetic interactions that should occur if a PBL-RacGAP50C complex is functionally important. The *RacGAP50C* RNAi phenotype is hypomorphic, as the severity of the phenotype increases with increased $RacGAP50C^{RNAi}$ expression (data not shown). When the $RacGAP50C^{RNAi}$ construct was driven by the *en::Gal4* driver at 25°C, cells of the posterior wing compartment were typically binucleate (see above), and the posterior wing morphology was disrupted (Figure 3B). Expression of a dominant-negative form of PBL carrying a deletion in the Dbl homology (DH) domain blocks cytokinesis when expressed in the developing eye (Prokopenko et al., 1999; O'Keefe et al., 2001), but shows essentially no phenotype when driven by *en::Gal4* at 25°C (Figure 3C). However, coexpression of the *RacGAP50C* RNAi and *pbl* dominant-negative constructs resulted in a dramatically enhanced phenotype, with complete loss of the posterior half of the wing blade (Figure 3D). This very strong genetic interaction suggests an important in vivo role for the PBL-RacGAP50C interaction. Significantly, it also shows that the two proteins act synergistically and not antagonistically, despite the fact that one is a Rho family GEF and the other is a Rho family GAP.

RacGAP50C and the Kinesin-like Motor Protein Pavarotti Form a Complex

Previous work has shown that *C. elegans* CYK-4 colocalizes with the kinesin-like motor protein ZEN-4/CeMKLP1, and that localization of these proteins to the central spindle is interdependent (Jantsch-Plunger et al., 2000). Furthermore, a multimeric complex between CYK-4 and ZEN-4, termed centralspindlin, is sufficient to bundle microtubules in vitro (Mishima et al., 2002). Cycle 16 cells mutant for *pavarotti* (*pav*), the *D. melanogaster* ortholog of *zen-4*, have a reduced level of microtubule bundling and show no cortical furrowing (Adams et al., 1998).

We therefore tested for an interaction between RacGAP50C and PAV using a yeast two-hybrid assay. We found that PAV interacted with RacGAP50C through N-terminal sequences adjacent to the PBL interaction domain (Figure 4A). To prove that RacGAP50C and PAV form an in vivo complex, we performed coimmunoprecipitation experiments. When embryonic extracts were immunoprecipitated with either polyclonal anti-RacGAP50C or anti-PAV antibodies, both the 70 kDa RacGAP50C (Figure 4B, upper panel) and 110 kDa PAV (Figure 4B, lower panel) proteins were detected. These data show that RacGAP50C and PAV are associated in vivo.

Coimmunostaining of S2 cells revealed that RacGAP50C and PAV colocalize throughout the cell cycle (Figure 4C). Both proteins decorate the mitotic spindle

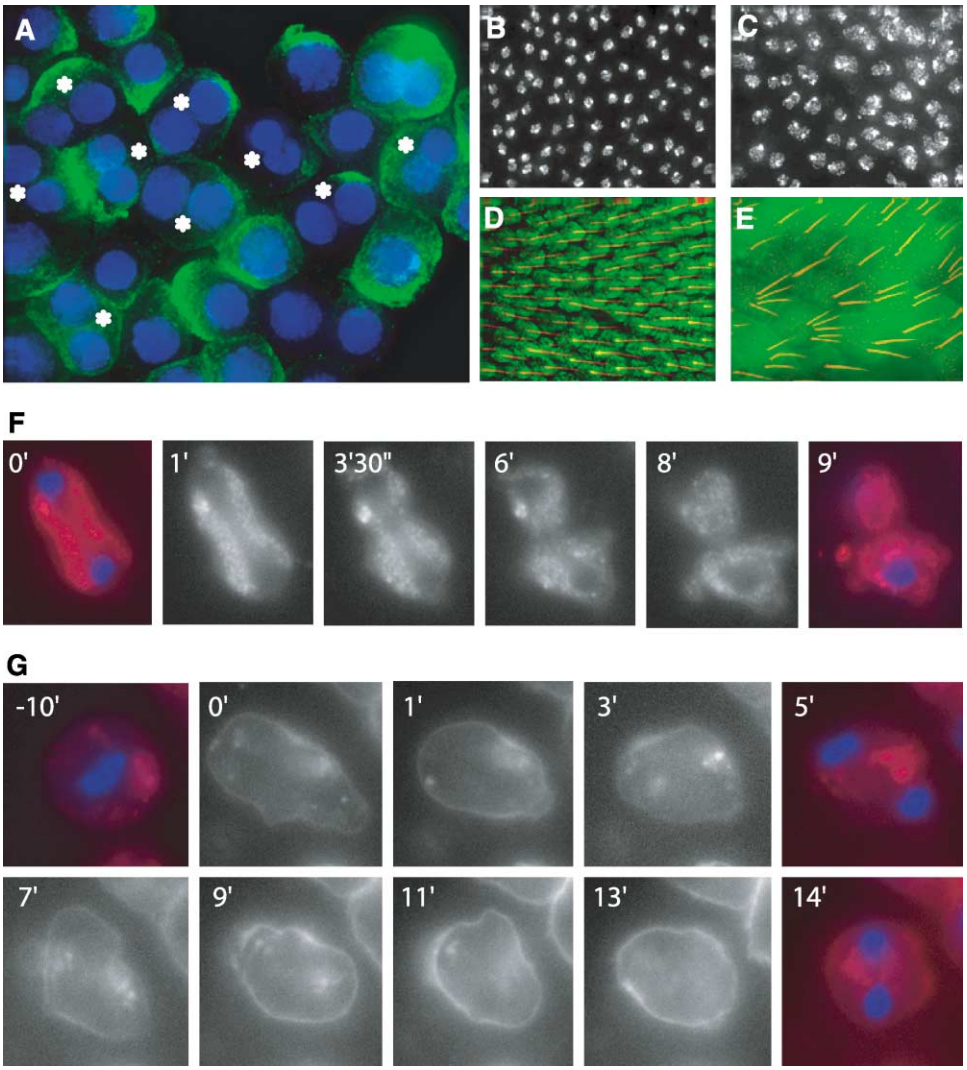


Figure 2. RacGAP50C RNAi Disrupts Cytokinesis

(A) Incubation of *D. melanogaster* S2 cells with double-stranded RNA derived from a RacGAP50C cDNA results in binucleate cells. DNA was stained with Hoechst 33258 (blue) and microtubules were stained with anti-α-tubulin (green). Binucleate cells are marked with asterisks.

(B–E) The morphology of wild-type (B and D) and *RacGAP50C RNAi*-expressing (C and E) pupal wing cells, 36 hr after pupal formation. Nuclei were stained with Hoechst 33258 (B and C), and apical cell boundaries, which are in the same field but in a different focal plane to the basal nuclei cell membranes, were outlined by expression of a *UAS::mCD8GFP* construct (green in [D] and [E]). Comparison of the number of nuclei and cells per field of view indicated that the *RacGAP50C RNAi* cells were typically binucleate. Rhodamine-phalloidin (red) was used to stain the prehairs (D and E).

(F and G) Images of living *D. melanogaster* Schneider line 2 (S2) cells undergoing mitosis. Black and white panels represent SynaptoRed staining of membranes, while colored panels represent Hoechst 33258 (blue) staining of DNA and SynaptoRed (red) staining. The times represent the time the image was taken after elongation of the cell during anaphase B.

(F) Untreated S2 cells during late anaphase and telophase show stable furrowing. One nucleus in the final frame is out of the plane of focus.

(G) Twenty-four hours after *RacGAP50C* RNAi treatment, cells show random deformations in shape but no evidence of furrow formation. The metaphase figure shown in the first image preceded the late anaphase-telophase series by 10 min.

Supplemental movies from which these figures are taken can be viewed at http://www.developmentalcell.com/cgi/content/full/4/1/29/DC1.

during metaphase, and then localize to the central spindle and midzone cortex (Figure 4C, arrows) during anaphase before becoming restricted to the midbody during telophase and accumulating in the nucleus in interphase (data not shown). We conclude that this GAP kinesin-like protein complex identified first in *C. elegans* is conserved in *D. melanogaster*.

Cortical PBL Colocalizes with an Equatorial Ring of Microtubule-Associated RacGAP50C-PAV Complexes during Cytokinesis

The analysis described above suggested that the PBL RhoGEF, RacGAP50C and the PAV kinesin-like protein are associated within dividing embryonic cells. To determine where this association occurs, we examined the

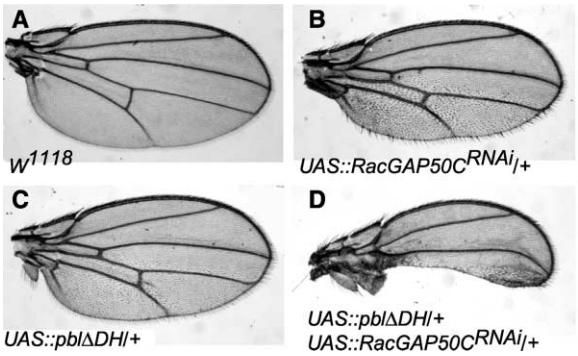


Figure 3. *pbl* and *RacGAP50C* Exhibit a Strong Synergistic Genetic Interaction

(A) A wild-type adult wing.

(B) Ectopic expression of the *RacGAP50C RNAi* construct driven by the *en::Gal4* enhancer resulted in disruption of the cellular morphology in the posterior half of the adult wing due to cytokinesis defects (see Figure 2).

(C) *en::Gal4*-induced expression of PBL deleted for a portion of the Dbl homology (DH) domain resulted in subtle abnormalities in posterior wing morphology.

(D) Coexpression of the *pbl* deletion construct and the *RacGAP50C RNAi* construct produced a strongly synergistic phenotype that disrupted formation of the posterior half of the wing.

subcellular distribution of the three proteins during embryonic cell divisions. PBL has previously been shown to accumulate cortically at the site of the contractile ring (Prokopenko et al., 1999), while PAV and the RacGAP50C homologs, CYK-4 and MgcRacGAP, associate with the mitotic spindle and then the central spindle and midbody (Adams et al., 1998; Jantsch-Plunger et al., 2000; Hirose et al., 2001; Mishima et al., 2002).

In dividing *D. melanogaster* embryonic epithelial cells, RacGAP50C was present at low levels on the mitotic spindle during metaphase, appeared as punctate dots in the region of the central spindle prior to furrowing during anaphase (Figure 5A, empty arrowheads), formed a more concentrated midzone band during the earliest stages of furrowing (solid arrowheads), and then localized to the midbody during late telophase (asterisks).

Although this localization pattern is consistent with that described for RacGAP50C orthologs, we observed one significant difference. Three-dimensional projections of late anaphase/early telophase cells revealed that the majority of the RacGAP50C-PAV complexes form a punctate cortical ring at the site of furrowing (Figure 5) where they abut or overlap the contractile ring, represented in Figure 5C by PBL and anillin. In some cells, the RacGAP50C/PAV ring appeared to precisely abut the intracellular face of the cortical PBL/anillin ring (Figure 5C). The association of RacGAP50C with the inner face of the contractile ring was also observed with the contractile ring components Peanut, myosin II, and the formin homology protein Diaphanous (data not shown). As expected, anti-α-tubulin staining revealed that the PAV-RacGAP50C complexes were associated with cortical microtubules (Figures 4C and 5B, panels a and b, and data not shown). PAV-RacGAP50C complexes were also found at the midzone of a bundled central set of microtubules present during anaphase (Figures 5A and 5B). We conclude that a double-ring structure, composed of actomyosin-associated contractile ring components and microtubule-associated PAV-RacGAP50C complexes, forms at cytokinesis onset in *Drosophila* epithelial cells.

PAV, but Not PBL, Is Required for Microtubule Midzone Localization of RacGAP50C

We next asked whether the association with PAV and/or with PBL was responsible for localization of RacGAP50C to the spindle midzone. First, we examined the localization of RacGAP50C in *pav* mutant embryos. We found that mitotic cycle 16 *pav* mutant cells were depleted for bundled microtubules and lacked RacGAP50C in the central spindle/midbody region (Figure 6A). In contrast, RacGAP50C was found to localize normally to the spindle midzone in *pbl*^{2} mutant embryos, even in the presence of the highly abnormal mitotic figures that are present in the later divisions (Figure 6B). We also found that S2 cells treated with *pbl* dsRNA possessed a central spindle with RacGAP50C correctly localized to the midzone region (Figure 6C). We conclude that RacGAP50C localizes to the spindle midzone in a PAV-, but not PBL-, dependent fashion.

Does RacGAP50C Require a Functional GAP Domain during Cytokinesis?

The cortical localization of the PAV-RacGAP50C complex places it in a position to regulate Rho family members through its GAP activity. However, the in vitro microtubule bundling activity demonstrated for this complex (Mishima et al., 2002) occurs in the absence of any Rho family small G proteins. We therefore asked whether the GAP domain was required for the function of *RacGAP50C* in cytokinesis. We used the fact that the *RacGAP50C*^{RNAi} phenotype was suppressed by coexpression of MYC-tagged wild-type RacGAP50C (data not shown). However, coexpression of MYC-RacGAP50C$^{\Delta EIE}$ and MYC-RacGAP50C$^{\Delta YRL}$, which each contains a targeted deletion of three essential catalytic residues in the GAP domain (Ahmed et al., 1994; Leonard et al., 1998), was unable to suppress the cytokinetic phenotype, despite expression of the construct being confirmed by anti-MYC staining (data not shown). While it is possible that these forms fail to rescue because of nonspecific disruption to their structure, these data suggest that the GAP domain is essential for the cytokinetic function of RacGAP50C.

In an attempt to find a potential target of RacGAP50C GAP function during cytokinesis, we carried out a number of genetic interaction experiments using the RacGAP50C RNAi phenotype and combinations of mutations in genes encoding Rho family members. Significantly, removing one copy of *Rho1* did not modify the phenotype, suggesting that Rho1 is not the target of RacGAP50C. Mutations in *Cdc42*, *Rac1*, *Rac2*, and *mtl*, and the *Rac1 Rac2 mtl* triple mutant had no effect on the *RacGAP50C RNAi* phenotype. If RacGAP50C does have a target small G protein during cytokinesis, either these haploinsufficiency tests are not sufficiently sensitive to identify it or the target is yet to be tested.

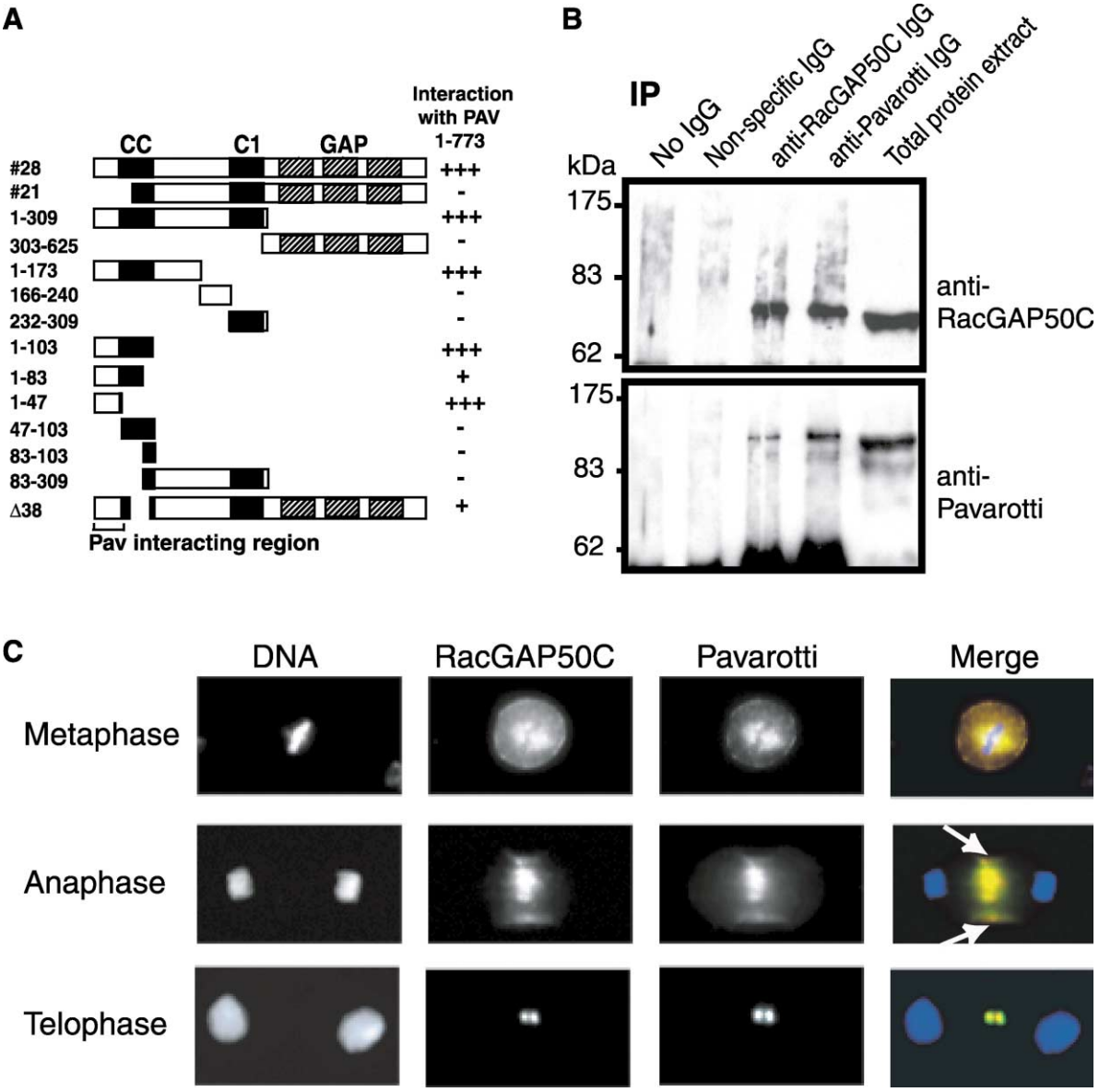


Figure 4. RacGAP50C Associates with the Pavarotti Mitotic Kinesin-like Protein

(A) Yeast two-hybrid assay results showing that the PAV interaction domain of RacGAP50C is situated at the extreme N terminus, adjacent to the coiled-coil PBL interacting domain. The constructs used are represented on the left and results of the interactions are indicated on the right, with +++ indicating a strong interaction, ranging down to −, which indicates no interaction. The domains are described in the legend to Figure 1.

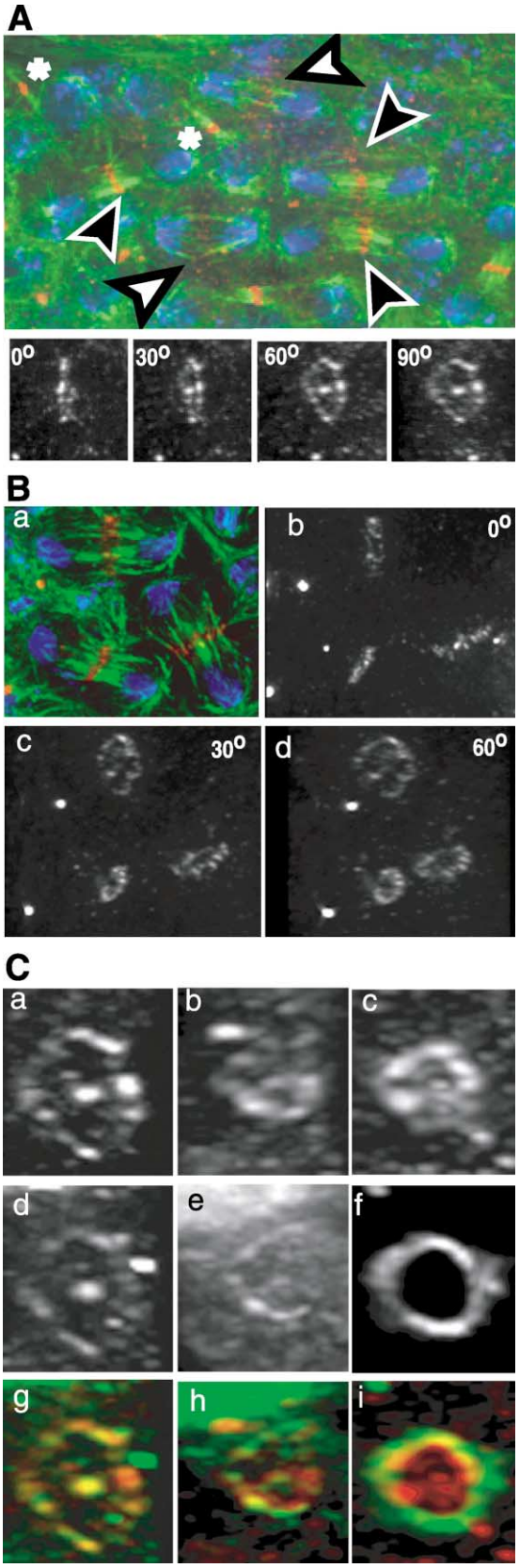
(B) RacGAP50C and PAV coimmunoprecipitate from embryonic extracts. Anti-RacGAP50C, anti-PAV, or nonspecific IgG immunoprecipitates of 0–5 hr embryo extracts were separated by SDS-PAGE and transferred to a nitrocellulose filter. The filter was probed with anti-RacGAP50C antibodies (top panel) and then stripped and probed with anti-PAV antibodies (bottom panel). Anti-PAV immunoprecipitated a 70 kDa RacGAP50C protein (upper panel, lane 4), and anti-RacGAP50C immunoprecipitated a 110 kDa PAV protein (lower panel, lane 3).

(C) RacGAP50C and PAV colocalize throughout the cell cycle in *D. melanogaster* S2 cells. In the merge panel, RacGAP50C is red, PAV is green, and DNA, stained with Hoechst 33258, is blue. Both proteins are predominantly found on the mitotic spindles during metaphase, the central spindle during anaphase, and the midbody during telophase. During anaphase, PAV and RacGAP50C are associated with the cortex (arrows).

Discussion

A Rho GTP Exchange Factor and Rho Family GTPase-Activating Protein Connect the Contractile Ring and Microtubule Networks at the Site of Furrowing during Cytokinesis

We have identified an interaction between the RhoGEF, Pebble (PBL), and a Rho family GAP, RacGAP50C, two conserved regulatory factors that are essential for cytokinesis. Yeast two-hybrid interactions, coimmunoprecipitation, subcellular colocalization, common cytokinetic phenotypes, and strong genetic interactions all support the existence and biological significance of this interaction. In *D. melanogaster* embryonic epithelial cells at the onset of cytokinesis, the two proteins are part of a cortical double-ring structure at the site of cleavage



furrowing. The RacGAP50C ring is associated with cortical microtubules, presumably through its interaction with the Pavarotti (PAV) kinesin-like protein. PAV colocalizes with RacGAP50C, and coimmunoprecipitation experiments showed that they form a complex in vivo. The RacGAP50C-PAV ring appears to abut or overlap the PBL-containing contractile ring (Prokopenko et al., 1999). The PAV kinesin-like protein, RacGAP50C and PBL RhoGEF complex simultaneously associates with, and has the capacity to control, both the actin and microtubule cytoskeletons as they are remodeled during cytokinesis. Furthermore, this complex appears to be a conserved feature of animal cytokinesis, as the mammalian PBL and RacGAP50C orthologs, the protooncogene ECT2 and MgcRacGAP, bind to each other in a yeast two-hybrid assay (this study) and colocalize during mitosis (Tatsumoto et al., 1999; Hirose et al., 2001).

We found that the interaction between PBL and RacGAP50C occurs through an extended BRCT domain of PBL and an N-terminal coiled-coil domain of RacGAP50C. RacGAP50C was found to bind PAV through sequences adjacent to the PBL-interacting domain, indicating the presence, in *D. melanogaster*, of the centralspindlin complex first identified from analysis of CYK-4 and ZEN-4, the *C. elegans* RacGAP50C and PAV orthologs (Mishima et al., 2002).

Formation and Localization of the RacGAP50C-PAV Ring

RacGAP50C-PAV complexes were found to be cytoplasmic at prophase, associated with mitotic spindles during metaphase, concentrated in the spindle midzone during anaphase, and localized to the midbody at cytokinesis and to the nucleus during interphase. During late

Figure 5. PAV Kinesin-like Protein, RacGAP50C, and PBL RhoGEF Complexes Form a Cortical Equatorial Ring during Cytokinesis

(A) Upper panel: anti-RacGAP50C (red) and anti-α-tubulin (green) staining reveals that RacGAP50C is associated with microtubules, appearing as punctate dots in the region of the cortical spindle prior to furrow ingression (white arrowheads), then in a more concentrated band at the spindle midzone later in anaphase and telophase (black arrowheads), and finally in the midbody during telophase (asterisks). DNA was stained with Hoechst 33258 (blue). Lower panel: a three-dimensional rotation, in 30° increments, of an anaphase/telophase mitotic figure stained with anti-RacGAP50C reveals localization of RacGAP50C to a cortical ring at the site of the spindle midzone and furrowing, as well as to the midzone of bundled central microtubules.

(B) A three-dimensional rotation of a z-series through late anaphase/telophase embryonic cycle 14 mitotic figures. Panel a: composite image of cells stained with anti-RacGAP50C (red), anti-α-tubulin (green), and the Hoechst 33258 DNA stain (blue). Panels b–d: the same field showing only the anti-RacGAP50C staining rotated about the vertical axis by 0°, 30°, and 60°, respectively, showing that the RacGAP50C ring contracts as cytokinesis proceeds.

(C) Double-labeling experiments reveal colocalization of RacGAP50C, PAV, PBL, and the contractile ring component anillin. Panels a–c show staining with anti-RacGAP50C, shown also in red in the merged figures (panels g–i). Panel d: anti-PAV staining, shown in green in the merge figure (panel g); panel e: anti-GFP staining of PBLGFP, shown in green in the merge figure (panel h); panel f: anti-anillin staining, shown in green in the merge figure (panel i).

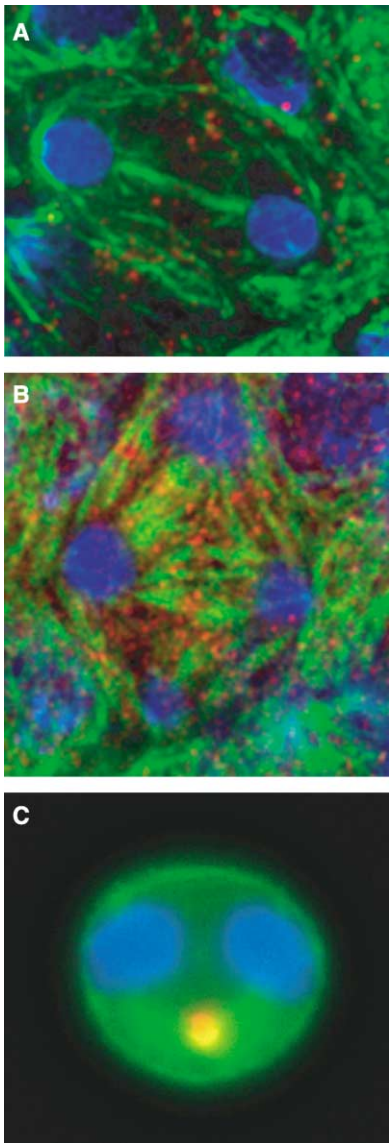


Figure 6. The PAV Kinesin-like Protein, but Not the PBL RhoGEF, Is Required for the Midzone Localization of RacGAP50C

D. melanogaster embryonic pav^{B200} (A), pbl^{2} (B), and *pbl* RNAi-treated S2 cells (C) stained with anti-RacGAP50C (red), anti-α-tubulin (green), and Hoechst 33258 (DNA; blue).

(A) A cycle 16 pav^{B200} mutant cell shows depleted microtubule bundling with no cortical or midzone localized RacGAP50C.

(B) A tetraploid cycle 15 telophase cell within a homozygous pbl^{2} mutant embryo displays a complex array of mitotic spindles (green) with RacGAP50C localizing to the midzone region of each set of spindles.

(C) An S2 cell treated with *pbl* double-stranded RNA forms abnormal spindles, with RacGAP50C localizing to the midzone of the mitotic spindle remnant.

anaphase and early telophase in *D. melanogaster* epithelial cells, RacGAP50C-PAV complexes not only localize to the overlapping microtubules of the centrally located anaphase spindle, but also to distinct cortical microtubules. Cortical microtubules have been reported in dividing *D. melanogaster* neuroblasts (Savoian and Rieder, 2002), and we observed them in all *D. melanogaster* anaphase cells examined. Localization of the RacGAP50C-PAV complexes to the microtubule midzone is independent of its interaction with PBL, as RacGAP50C is found to localize appropriately in PBL mutant cells. However, localization of RacGAP50C is dependent on the PAV kinesin-like protein. The affinity of the RacGAP-KLP complex for microtubules, the cortical localization of the microtubules, and the plus end-directed nature of the PAV kinesin-like motor protein appear sufficient to account for localization of the complex to an equatorial cortical ring.

A Molecular Model for Positioning the Contractile Ring

The molecular signal that positions the contractile ring and initiates furrowing remains to be elucidated. A number of studies have shown that the signal derives from the overlapping midzone microtubules that form during anaphase (Wheatley and Wang, 1996; Cao and Wang, 1996). One of the most striking aspects of the formation of the PBL-RacGAP50C ring is that it is present in the earliest examples of furrowing that we have observed. The existence of this ring at the onset of cytokinesis allows us to propose a molecular model for the positioning and regulation of the contractile ring (Figure 7). Specifically, we propose that the initiation signal corresponds to the microtubule-mediated arrival of the RacGAP50C-PAV kinesin-like protein complex at its equatorial ring and establishment of the interaction with the PBL RhoGEF. We propose that this interaction results in activation of RhoGEF activity. Rho1 would then be activated to initiate contractile ring formation and furrowing through activation of factors such as Diaphanous and myosin. This model accounts for the role of microtubules in positioning the contractile ring, because microtubules deliver the RacGAP50C-PAV complexes to their interaction site with PBL. It also accounts for the conclusion, made by Gatti and colleagues from their studies of cytokinesis, that there is a requirement for both the central spindle and a cortical PBL-containing apparatus for the onset of cytokinesis (see, for example, Somma et al., 2002).

There is strong support for this model beyond the evidence described here. Importantly, consistent with our observation that *DRacGAP50C* RNAi-treated S2 cells show no furrowing, *pav* and *pbl* mutant cells fail to form a contractile ring and do not undergo furrow ingression (Adams et al., 1998; Prokopenko et al., 1999). However, contradictory evidence has come from *C. elegans*, where *cyk-4* and *zen-4* mutant cells initiate but fail to complete furrowing (Raich et al., 1998; Jantsch-Plunger et al., 2000). We suggest two possible explanations for these contradictory observations. The first is that *D. melanogaster* epithelial cells may use a different cytokinesis mechanism than that used in the early *C. elegans* embryo. In support of this, the cell types are very different in size and exhibit differences in microtubule organization during anaphase and telophase. Alternatively, it is possible that the *C. elegans* phenotypes do not represent the true null phenotype. The *cyk-4* allele used to determine the phenotype is a temperature-sensitive allele (*cyk-4(t1689ts)*; Jantsch-Plunger et al., 2000), which may not abolish all activity at the restrictive temperature. The *zen-4* allele used to generate germline

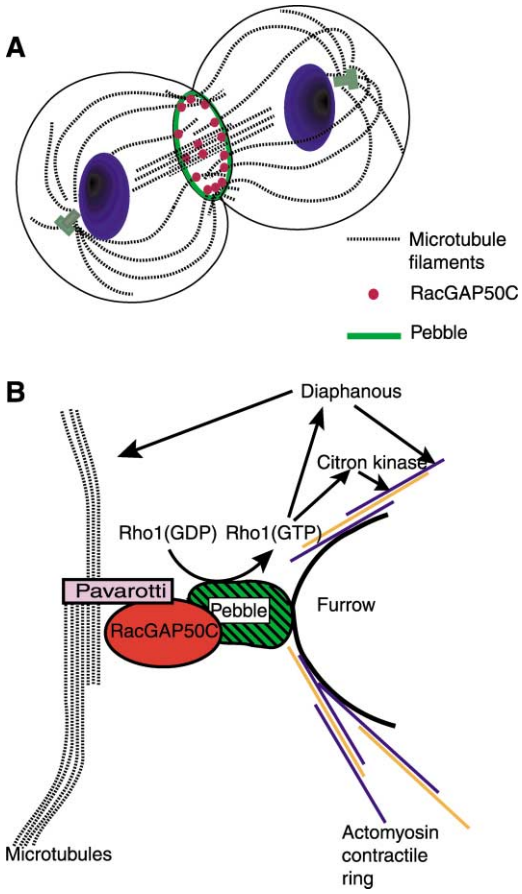


Figure 7. The Pav-RacGAP50C-PBL Complex Provides a Molecular Explanation for the Positioning and Regulation of Furrowing

We propose that the PAV-RacGAP50C-PBL complex positions the contractile ring and coordinates cytoskeletal remodeling during cytokinesis.

(A) The site of contractile ring formation is specified by localization of RacGAP50C to the spindle midzone through the activity of its binding partner, the plus end-directed molecular motor, PAV.

(B) At this site, the PBL RhoGEF-RacGAP50C-PAV kinesin-like protein complex forms, resulting in the activation of Rho1 and therefore of contractile ring function through its regulation of factors such as the FH protein, Diaphanous, and citron kinase.

mutant clones is a premature truncation that would eliminate all function. However, to observe the phenotype, germline clones were generated, perhaps requiring *zen-4* activity to undergo the previous division. Some of the product may therefore have persisted to produce the partial furrowing observed in the mutant embryos. It therefore remains to be seen whether the model proposed here is applicable to cytokinesis in all animal cells.

PBL and RacGAP50C Are Important Regulators of the Cytoskeleton and Cytokinesis

Midzone microtubule bundles have been shown to be required continuously for cytokinesis in cultured cells (Wheatley and Wang, 1996). The cortical PBL-RacGAP50C-PAV ring, which persists and narrows as cytokinesis proceeds, is ideally positioned to coordinate actomyosin contraction and the bundling of microtubules. Actin filament activity is regulated by PBL, which is required for establishment and/or maintenance of the contractile ring through activation of the Rho1 GTPase (Prokopenko et al., 1999). Microtubule bundling activity has been demonstrated for CYK-4 and ZEN-4 (Mishima et al., 2002). It is likely, therefore, that the complex between the PBL RhoGEF, RacGAP50C and the PAV kinesin-like protein functions to coordinate F-actin and microtubule remodeling during contractile ring constriction.

While actomyosin regulation and microtubule bundling may be the primary regulatory roles of these factors, there are additional ways that the PBL RhoGEF and RacGAP50C could influence both the actin and microtubule-based cytoskeleton. Rho downstream effectors have been shown to regulate both cytoskeletal systems. For example, the Rho1 target, Diaphanous, mediates actin reorganization (Watanabe et al., 1999) but also affects the stability of microtubules (Palazzo et al., 2001).

RacGAP50C Regulation of Small G Protein Activity

The CYK-4 and ZEN-4 microtubule bundling activity does not require the presence of any of the small G proteins, but the site-directed mutant analysis described here suggests a requirement for the GTPase-activating domain of RacGAP50C. Consistent with this, a GAP domain-defective form of MgcRacGAP appears to act as a dominant-negative protein, inducing cytokinetic defects (Hirose et al., 2001). If such a target of RacGAP50C GAP activity exists, we have not been able to identify it. However, our evidence is inconsistent with RacGAP50C acting as the Rho1 GAP that opposes PBL, based on the synergistic nature of *pbl* and *RacGAP50C* genetic interactions and on the absence of genetic interactions between *RacGAP50C* and *Rho1*. Consistent with this, in vitro assays show that the CYK-4 and MgcRacGAP homologs target Rac and Cdc42 with far greater efficiency than Rho1 (Jantsch-Plunger et al., 2000; Touré et al., 1998; Kawashima et al., 2000).

Conclusion

We have identified complexes between the RhoGEF PBL and the Rho family GAP, RacGAP50C, and between RacGAP50C and the kinesin-like protein, PAV, that connect the contractile ring to cortical microtubules during cytokinesis. During late stages in anaphase and during telophase, these proteins localize to a cortical ring where furrowing is initiated, constricting as furrowing proceeds. Our observations suggest a model for the molecular control of cytokinesis in animal cells, whereby microtubule-dependent cortical equatorial localization of RacGAP50C-PAV kinesin-like protein complexes is the positioning signal generated by the central spindle microtubules, and formation of complexes with the PBL RhoGEF allows coordination of F-actin and microtubule remodeling.

Experimental Procedures

Yeast Two-Hybrid Experiments

The yeast two-hybrid plasmid vectors pVP16 and pLexA-NLS (pNLX) (Hollenberg et al., 1995), a 0–4 hr *Drosophila* embryonic cDNA library in pVP16, and the yeast strain L40 (MATa his3Δ200 trp1-901 leu2-3,112 ade2 LYS2::$(lexAop)_4$-HIS3 URA3::$(lexAop)_8$-lacZ GAL4 gal80),

were kindly provided by Dr. S. Parkhurst, Fred Hutchinson Cancer Research Center, Seattle. An N-terminal $PBL_{1–308}$ peptide fused to the LexA DNA binding domain was used as a bait in the yeast two-hybrid screen. Other LexA/PBL fusion protein constructs generated by PCR amplification correspond to amino acids 1–101, 1–206, 94–206, 94–308, 197–308, 369–651, 369–853, and 571–853 of PBL. RacGAP50C constructs fused to the VP16 activation domain correspond to amino acids 1–309, 303–625, 1–173, 166–240, 232–309, 1–103, 1–83, 1–47, 47–103, 83–103, and 83–309 of RacGAP50C. The deletion construct RacGAP50CΔ38, which removes amino acids 66–103, was generated by site-directed mutagenesis (QuickChange, Stratagene). The entire ORF of *pavarotti* was PCR amplified from the EST LD24535 (ResGen) and cloned in-frame to the LexA DNA binding domain. The entire ORF of MgcRacGAP was PCR amplified from a clone kindly provided by Dr. T. Kitamura (University of Tokyo) and cloned in-frame with the LexA DNA binding domain. The ECT2 N terminus (amino acids 1–333) was PCR amplified from a human testis cDNA library (Zap Express XR, Stratagene) and cloned in-frame into the VP16 activation domain.

Antibodies

Primary antibodies used included mouse anti-α-tubulin (Sigma), rabbit anti-PAV (Rb3301; Adams et al., 1998; kindly provided by Professor D. Glover, University of Cambridge), rat anti-PBL (R9; Prokopenko et al., 1999), and rabbit anti-GFP (Molecular Probes). Anti-anillin, anti-Peanut, anti-myosin II, and anti-Diaphanous were all kindly supplied by Dr. C. Field (Harvard Medical School). Secondary antibodies and tertiary complexes (Jackson Immunoresearch Laboratories) included Alexa 488-conjugated donkey anti-mouse, rhodamine-conjugated donkey anti-rat, goat anti-rat biotin, goat anti-rabbit biotin, Alexa 488-conjugated streptavidin, and rhodamine red-conjugated streptavidin. Horseradish peroxidase (HRP)-conjugated donkey anti-rabbit and goat anti-rat antibodies were used for Western blot detection. Polyclonal rabbit anti-RacGAP50C antibodies were generated against full-length RacGAP50C fused to GST. IgG was purified through protein G HiTrap columns (Amersham Pharmacia).

Double-Stranded RNA Synthesis, RNAi Treatment, Cell Culture, and Live Imaging

cDNAs encoding PBL, RacGAP50C, and the Rho family GTPases Rho1, RhoL, Rac1, Rac2, Cdc42, and Mtl (kindly provided by Dr. B. Dickson, IMP, Vienna) were transcribed in both directions using T7 RNA polymerase. The mixture was DNase treated, incubated at 100°C for 1 min, and the transcripts annealed by slow cooling to room temperature. *D. melanogaster* Schneider line 2 (S2) cells were grown in 1× Schneider's *Drosophila* media (GIBCO) supplemented with 10% foetal calf serum (FCS) at 25°C. RNAi treatment of S2 cells was carried out as previously described (Clemens et al., 2000), using 10–20 μg of annealed RNA. After treatment, the cells were allowed to grow at 25°C for the times indicated in the text before being fixed and stained (see below) or imaged live using a DeltaVision deconvolution microscope. For live imaging, cells were incubated for 5 min in Schneider's medium containing the vital stains Hoechst 33258 (1 μg/ml) to stain DNA, and SynaptoRed (Sigma; 0.5μg/ml) to stain membranes, before the cells were returned to Schneider's medium.

Immunofluorescence Analysis

S2 cells adhered to coverslips were washed several times with phosphate buffered saline (PBS), and fixed with 3.7% formaldehyde (Sigma) in PBS plus 0.1% Tween-20 (PBT; Sigma) for 15 min at room temperature. The cells were washed, blocked in PBT with 0.2% BSA and 5% normal goat serum for 30 min at 37°C and then incubated with primary antibodies in the same block for 1.5 hr at 37°C. They were then washed in PBS and incubated with secondary antibodies for 1.5 hr at 37°C, before being washed in PBS. The cells were treated with Hoechst 33258 (10 μg/ml), washed, and mounted in 80% glycerol. Immunofluorescent staining of *D. melanogaster* embryos was carried out according to the method of Tautz and Peiffer (1989). Specimens were analyzed using an epifluorescence Olympus microscope or a Delta Vision (Applied Precision) deconvolution microscopy system and micrographs prepared with Adobe Photoshop.

Pupal Wing Analysis

Pupal wings were dissected and stained using the method of Winter et al. (2001). Thirty-six hour APF wings were dissected and fixed in 4% formaldehyde in PBT. F-actin was detected with 200 nM Phalloidin-TRITC (Sigma).

Immunoprecipitation

Staged embryos were homogenized on ice in 50 mM Tris/HCl (pH 7.5), 1 mM EDTA, 0.1% NP-40 with Complete Mini protease inhibitors (Roche) and spun at 14,000 rpm for 10 min at 4°C. Appropriate antibodies were added to solubilized protein and incubated with mixing at 4°C for 3 hr. One hundred microliters of protein A Sepharose TM CL-4B (Amersham Pharmacia) was added and incubated for a further 3 hr at 4°C. The Sepharose was pelleted, washed with homogenization buffer, resuspended in protein reducing buffer, boiled for 5 min, pelleted, and the supernatant electrophoresed in an SDS-polyacrylamide gel. The separated proteins were transferred to nitrocellulose (Schleicher and Schuell), and probed with anti-RacGAP50C or anti-PAV antibodies. Detection was performed with HRP-conjugated anti-rat or anti-rabbit secondary antibodies and ECL reagent (Amersham Pharmacia).

Fly Stocks and Transgenic Constructs

The *UAS::MYC-RacGAP50C* construct was generated by PCR amplification of the entire ORF of the full-length yeast two-hybrid clone. The PCR product was cloned into the pCS2+MT vector to generate a construct that encoded an in-frame amino-terminal MYC tag fusion protein. This construct was subcloned into *pUAST* (Brand and Perrimon, 1993). Site-directed mutagenesis was used to generate *RacGAP50C*$^{\Delta EIE}$ and *RacGAP50C*$^{\Delta YRL}$, which encoded proteins lacking amino acids 404–406 and 416–418, respectively. The mutations were confirmed by sequence analysis and transformed lines were generated. The *UAS::RacGAP50C*^{RNAi} construct was generated by a head-to-head cloning of two overlapping PCR amplification products that, when transcribed, produced a foldback region of double-stranded RNA corresponding to nucleotides 248–1125 of the *RacGAP50C* open reading frame.

Other fly stocks used in this study were *UAS::pbl*$^{\Delta DH}$ (Prokopenko et al., 1999), *UAS::pbl-GFP* (P. Smibert and R.S., unpublished observations), *engrailed::Gal4* (Bloomington Stock Centre), *Cdc42*^{4} (Fehon et al., 1997), *pbl*^{2} (Jürgens et al., 1984), *Rho1*^{72O} (Strutt et al., 1997), *Rac1*^{J11}, *Rac2*$^{\Delta}$, *Mtl*$^{\Delta}$, and a *Rac1*^{J11} *Rac2*$^{\Delta}$ *Mtl*$^{\Delta}$ triple mutant stock (Hakeda-Suzuki et al., 2002; Ng et al., 2002), and *UAS::mCD8-GFP* (Winter et al., 2001).

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